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Possible Use of 'Alcaligenes paradoxus' as a Biological Monitor

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Possible Use of *Alcaligenes paradoxus* as a Biological Monitor

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16. ABSTRACT

A tritium ($^3H_2$)-oxidizing soil isolate was identified as *Alcaligenes paradoxus*, a gram-negative, rod-shaped bacterium. This organism belongs to a group of facultative autotrophs referred to as the "hydrogen bacteria" due to their unique ability to utilize hydrogen as a sole source of energy for chemolithotrophic growth.

Experiments with washed cells of *A. paradoxus* suspended in 0.025M potassium phosphate buffer (resting cells) showed that 1.0 ppm mercury [as $Hg(NO_3)_2$] caused a 95 percent reduction in hydrogen oxidation, whereas, concentration of 0.1 ppm and lower showed no inhibitory effects. When suspensions of *A. paradoxus* were added to sterile soil and then amended with mercury at concentrations of 1.0, 10.0, and 100.0 ppm, hydrogen oxidation was reduced 80 percent, 85 percent, and 95 percent, respectively, compared to soil controls with no mercury added. This showed that soil afforded, at least temporarily, some protection from toxic mercury effects.

When cadmium or lead was used in solution, it was found that up to 100.0 ppm of these metals produced no detectable inhibition in the rate or extent of the hydrogen oxidation reaction at either pH 7.0 or 5.0. However, pretreatment of *A. paradoxus* cells with a combination of ethylenediaminetetraacetate (EDTA) and tris-(hydroxymethyl)-aminomethane (Tris), at a pH of 8.0, resulted in a reduction in oxidation in solutions containing 10.0 ppm of cadmium or lead, with the greater reduction being due to cadmium.

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POSSIBLE USE OF *ALCALIGENES PARADOXUS* AS A BIOLOGICAL MONITOR

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FOREWORD

Protection of the environment requires effective regulatory actions that are based on sound technical and scientific information. This information must include the quantitative description and linking of pollutant sources, transport mechanisms, interactions, and resulting effects on man and his environment. Because of the complexities involved, assessment of specific pollutants in the environment requires a total systems approach that transcends the media of air, water, and land. The Environmental Monitoring and Support Laboratory-Las Vegas contributes to the formation and enhancement of a sound monitoring data base for exposure assessment through programs designed to:

- develop and optimize systems and strategies for monitoring pollutants and their impact on the environment
- demonstrate new monitoring systems and technologies by applying them to fulfill special monitoring needs of the Agency's operating programs

This report is concerned with the development of a method to detect the bioavailable levels of pollutants in environmental samples. In this study the possible use of the bacterium, *Alcaligenes paradoxus*, to rapidly and cost-effectively detect low levels of mercury, cadmium, and lead is examined. The results from this preliminary research should be useful in the further development of this and other biological monitors and the assessment of the actual biological hazard posed by different toxic substances. This report should be valuable to all persons involved in the research and regulation of toxic substances whether in government, industry, or university programs.

George B. Morgan
Director
Environmental Monitoring and Support Laboratory
Las Vegas

PREFACE

The existence of the so-called "hydrogen bacteria" has been known for many years, and a considerable number of investigations into their properties have been conducted. These bacteria represent a group of facultative chemolithotrophs which obtain their energy from the oxidation of hydrogen and use this energy for the assimilation of carbon dioxide and other metabolic processes.

Their heterotrophic growth can occur at the expense of a wide variety of organic compounds. These bacteria differ from heterotrophic hydrogen-oxidizing microorganisms by virtue of their ability to use hydrogen as a sole source of energy for autotrophic growth while the others require added organics and thus are obligate heterotrophs.

The studies described herein came about as a result of the metabolic activity of some of these organisms. In studies on the oxidation of elemental tritium ($^3H_2$) by plants, it was discovered that the reaction was rapidly occurring in soil without plants. Tritium is the isotope of hydrogen with two added neutrons and is radioactive due to its spontaneous decay ($t_{\frac{1}{2}}$ = 12.3 years) and emission of a weak beta particle. This oxidation of elemental tritium was found not to occur in autoclaved soils, and subsequent work led to the isolation of a bacterium capable of carrying out the reaction in pure culture. This bacterium was later determined to belong to the previously mentioned group of "hydrogen bacteria."

Subsequent research into the properties of this organism was done and fell into four main areas into which the body of this report has been divided:

1. Taxonomic Studies
2. Growth Studies
3. Resting Cell Studies
4. Heavy Metal Effect Studies

Initially, interest was directed towards determining which organism was capable of rapidly oxidizing the tritium; but as the properties of the bacterium were defined, it was realized that this organism had the potential for use as a biological indicator of selected pollutants. The growth and resting cell studies resulted from a need for baseline data prior to initiation and understanding of those with the heavy metals.

SUMMARY

A tritium ($^3H_2$)-oxidizing soil isolate was identified as *Alcaligenes paradoxus*, a gram-negative, rod-shaped bacterium. This organism belongs to a group of facultative autotrophs referred to as the "hydrogen bacteria" due to their unique ability to utilize hydrogen as a sole source of energy for chemolithotrophic growth.

Growth studies showed that *A. paradoxus* had autotrophic doubling times from 5.0 to 6.0 hours when grown in a mineral salts medium plus an atmosphere of 70 percent H_2, 20 percent O_2, and 10 percent CO_2. The optimal pH for growth was found to be near 7.0 although growth occurred over the range from 5.0 to 9.0. No growth occurred at pH 4.0 or 10.0. The addition of glucose, fructose, leucine, or trypticase soy broth (TSB) to the autotrophic medium (pH 7.0) stimulated growth (except for leucine in one instance) and shortened the doubling times to 4.57, 3.07, 4.36, and 1.77 hours, respectively, when added at the time of inoculation with the cells. When these same organics were added after the cells had been allowed to undergo two autotrophic divisions, glucose, fructose, and TSB produced doubling times of 5.38, 4.12, and 2.40 hours after little or no lag period.

Experiments with washed cells of *A. paradoxus* suspended in 0.025M potassium phosphate buffer (resting cells) showed that the range of pH's over which hydrogen (tritium) oxidation occurred was from 4.0 to 9.0. The optimal rate and extent of the reaction occurred at pH 7.0.

Further studies with resting cells showed that 1.0 ppm mercury [as $Hg(NO_3)_2$] caused a 95 percent reduction in hydrogen oxidation, whereas, concentration of 0.1 ppm and lower showed no inhibitory effects. When suspensions of *A. paradoxus* were added to sterile soil and then amended with mercury at concentrations of 1.0, 10.0, and 100.0 ppm, hydrogen oxidation was reduced 80 percent, 85 percent, and 95 percent, respectively, compared to soil controls with no mercury added. This showed that soil afforded, at least temporarily, some protection from toxic mercury effects.

When cadmium or lead was used in solution, it was found that up to 100.0 ppm of these metals produced no detectable inhibition in the rate or extent of the hydrogen oxidation reaction at either pH 7.0 or 5.0. However, pretreatment of *A. paradoxus* cells with a combination of ethylenediaminetetraacetate (EDTA) and tris-(hydroxymethyl)-aminomethane (Tris), at a pH of 8.0, resulted in a reduction in oxidation in solutions containing 10.0 ppm of cadmium or lead, with the greater reduction being due to cadmium. These results suggested that although the cells may initially be insensitive to certain compounds, their sensitivity may be enhanced thus making them more suitable for potential future development as bioindicators of pollutants.

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INTRODUCTION

The increased awareness in recent years of the hazards presented by the deposition of toxic substances in the environment has stimulated research into methods for detecting their presence and the biological threat these pollutants pose. As a result we can now measure low levels of many toxic compounds in environmental samples, and much progress has been made in identifying the biological effects these substances cause. However, problems still exist in the determination of the actual bioavailable levels of many pollutants. Also rapid assessment of environmental samples for their biological toxicity is presently not possible. Plant or animal studies are generally slow and expensive.

Described herein are preliminary studies of a method to determine rapidly and cost-effectively the bioavailability of certain inorganic or organic pollutants by observing their inhibitory effect on the easily monitored enzymatic oxidation of hydrogen gas [using tritium ($^3H_2$) as a tracer] by the bacterium, *Alcaligenes paradoxus*.

Since very little information was available on this microorganism, it was necessary to do a number of background studies prior to those using pollutants. Therefore, this report has been divided into four sections starting with studies to determine if the microorganism would suffice for the wide variety typically encountered in environmental samples and finishing with toxic heavy metal effects.

The first section deals with the identification of the bacterium after it was isolated from a soil sample. The second is concerned with the rate of growth both autotrophically and heterotrophically of the microorganism. In the third section, the ability of *A. paradoxus* to carry out the enzymatic conversion of tritium to tritiated water under a variety of conditions is examined. In section four the effect on the microorganism of the previously mentioned pollutants, mercury, cadmium, and lead, is studied.

CONCLUSIONS AND RECOMMENDATIONS

The results of studies with the tritium-oxidizing bacterium, *Alcaligenes paradoxus*, indicate that it may be useful as a biological monitor for available levels of certain toxic substances. This research has demonstrated the *A. paradoxus* sensitivity to mercury, lead, and cadmium.

The ability of *A. paradoxus* to rapidly convert elemental tritium (hydrogen) to tritiated water and the ease with which the reaction can be followed

present a simple, effective method for assessing the concentration and effect of certain pollutants on a living organism. The autotrophic mode of existence of *A. paradoxus* obviates the need for organic supplements and the reaction with tritium will occur rapidly with cells suspended in buffer or soil. Additionally, although the presence of certain organics can stimulate the rate of growth of *A. paradoxus* under autotrophic conditions, these compounds do not appear to have a significant effect on the ability of *A. paradoxus* to carry out the oxidation reaction.

The wide range of pH's that can support both autotrophic growth and the oxidation reaction further increases the versatility of this microorganism. Thus, it should be possible to monitor the rate of tritium oxidation by *A. paradoxus* in samples as diverse as beach sand to peat bog soil and distilled water to untreated sewage.

The ability of 1.0 ppm mercury to inhibit almost completely the oxidation reaction and the development of a method for increasing its sensitivity to lead and cadmium at the 10.0 ppm level as shown in preliminary experiments, strongly suggests that future studies into the feasibility of utilizing *A. paradoxus* and/or similar organisms for the detection of biologically available levels of selected pollutants be given careful consideration.

Future standardization of technique and research into contributing factors can almost certainly make such organisms as *A. paradoxus* extremely reliable, inexpensive, and rapid indicators of the bioavailable levels of toxic substances in the environment.

SECTION I. TAXONOMIC STUDIES

INTRODUCTION

The hydrogen bacteria have been found to comprise a diverse group of microorganisms spanning a number of genera. At one time they were all relegated to the genus *Hydrogenomonas* due to their ability to utilize molecular hydrogen as a source of energy for chemolithotrophic growth (Bergey's manual, 1957). However, this was later found to be a poor basis of classification since many of these bacteria had so few other characters in common (Davis, 1969). In addition, the defining character of the genus, namely the ability to grow autotrophically at the expense of hydrogen could even be lost from some of these organisms after repeated cultivation with organic substrates (Kluyver and Manten, 1942; Packer and Vishniac, 1955; Wilson et al., 1953).

As a result of these observations the various hydrogenomonads were relegated to other genera more appropriately suited to them based on their other properties. These genera included *Pseudomonas*, *Alcaligenes*, and *Paracoccus*. Subsequent work has resulted in the isolation of hydrogen bacteria belonging to still more genera including *Nocardia* (Aggag and Schlegel, 1973); *Corynebacterium* (Andressen and Schlegel, 1974); *Mycobacterium* (Park and DeCicco, 1974); and *Streptomyces* (Takamiya and Tubaki, 1956).

The isolate used in this study was isolated by enrichment from soil and identified by means of the taxonomic criteria established for the identification of many of the hydrogen bacteria (Davis et al., 1970).

The following will detail the numerous morphological, biochemical, and physiological tests used in establishing the identity of the tritium-oxidizing isolate.

MATERIALS AND METHODS

The soil isolate was obtained in the following manner (Rogers et al., 1978). Mineral salts plates were prepared with:

KH_2PO_4	0.8 grams (g)
K_2HPO_4	0.8 g
$MgSO_4 \cdot 7H_2O$	0.2 g
$CaCl_2$	0.002 g
$FeSO_4 \cdot 7H_2O$	0.001 g
$NaNO_3$	4.0 g
Distilled water	1000 milliliters (ml)

The media were solidified by the addition of 1.5 percent Noble agar (Difco) and sterilized by steam autoclaving. These plates were then inoculated with various dilutions of a silty clay loam soil and put in a BBL (Baltimore Biological Labs) gas pack that generated an atmosphere of hydrogen and carbon dioxide. The packs are designed for the cultivation of anaerobes, but by removing the oxygen scavenger disc it was possible to obtain an atmosphere with oxygen and the aforementioned gases.

A variety of colonies appeared on the plates after approximately 10 days incubation at room temperature. Representative colonial types were selected and pure cultures obtained. Not all microorganisms that appeared were capable of hydrogen oxidation but, as determined by later experiments, one yellow colony (several strains of which were isolated) and an apparent streptomycete were the only active tritium-oxidizers isolated from the plates.

The yellow bacterial isolates were initially chosen for further investigation and, due to the success obtained with them, the streptomycete was not examined further.

After tests with other autotrophic media, it was found that the use of Repaske's media (1966) produced the best growth; and it was used thereafter for maintenance of all autotrophic cultures. It was also found that the use of an atmosphere of 70 percent hydrogen, 20 percent oxygen, and 10 percent carbon dioxide (Repaske et al., 1971) provided satisfactory gaseous conditions. After the initial isolation, subsequent cultures were maintained in dessicator jars which were evacuated, then refilled with the above gas mixture.

Determinations of the ability of the organism to utilize organic compounds as a sole source of carbon and energy were conducted according to

the procedures described by Stanier et al. (1966). All chemicals used were of reagent grade quality.

To examine isolates for the presence of cytochrome oxidase, a 1-percent solution of N,N,N^1,N^1 tetramethyl-p-phenylenediamine dihydrochloride was prepared in distilled water. A small strip of filter paper was impregnated with this solution and then a colony of the isolate was picked with a Pasteur pipette and rubbed on the filter paper. Development of a dark color within 20 seconds was considered a positive reaction.

The test for the presence of catalase was performed by dripping a 3-percent solution of hydrogen peroxide on colonies on nutrient agar. Bubbling was a positive response.

Motility and sulfide and/or indole production were determined using SIM media (Difco).

Nitrate reduction was determined by removing to separate wells small aliquots from a culture grown in a nitrate broth then adding a drop of sulfanilic acid solution [sulfanilic acid, 0.8 g, acetic acid (5N), 100 ml], followed by a drop of dimethyl-α-naphthylamine [N,N-dimethyl-1-naphthylamine, 0.5 g, acetic acid (5N), 100 ml]. The development of a red color indicated the presence of nitrite.

Denitrification was tested for using nitrate broth which was inoculated with the test organism and allowed to incubate for over 1 week. Gas formation within Durham tubes was considered positive for denitrification.

The ability to hydrolyze starch was checked by streaking organisms on nutrient agar plates supplemented with 0.2 percent weight/volume soluble starch then flooding the plates with Gram's iodine solution after good growth had occurred. Hydrolysis would be represented by clear areas around colonies.

Flagellation was determined from young cultures grown on trypticase soy agar (TSA) slants (BBL). Slides used for the test were soaked 1 week in freshly prepared dichromic acid. The stain was freshly prepared from BBL flagella stain. The procedure consisted of adding a 1- to 3-ml amount of distilled water to the slants thus obtaining a suspension of the cells in the water. Some of this water was then gently removed with a Pasteur pipette and 1 drop allowed to run down the length of several dry glass slides. When the solution had dried, the slides were stained as per the instructions supplied by BBL.

The test for the presence of indole was done using trypticase soy broth cultures in test tubes that were layered over with 1.0 ml of Kovac's reagent [(p-dimethylaminobenzaldehyde, 5 g; amyl alcohol, 75 ml; hydrochloric acid (conc.), 25 ml)], shaken gently, then observed for the development of a red color in the reagent which rises to the top.

Dry weight determinations were conducted by first growing the bacterial isolate under autotrophic conditions in the medium described by Repaske (1966) to an optical density of 0.35 as determined with a Bausch & Lomb Spectronic 20

colorimeter set at 540 nanometers (nm). Aliquots of 10.0 ml were then removed and centrifuged for 20 minutes at 8,000 x g. The supernate was discarded and the cells were resuspended in a small volume of distilled water, then recentrifuged as before. The water was decanted and the packed cells were carefully washed out of the centrifuge tubes into preweighed tins and dried at 65° C for 24 hours before being reweighed. Dilution plates were prepared to determine cell numbers in the culture media at the time the samples were removed. The dilutions were plated out on TSA since it had been determined by earlier tests that dilutions of autotrophic cell cultures actually yielded higher cell counts on the TSA than on autotrophic plates.

RESULTS AND DISCUSSION

Because the bacterial isolates all gave identical reactions, they were considered to be the same organism. The organism was found to be a gram-negative, yellow-pigmented rod capable of tenacious, slimy growth under autotrophic conditions.

Table 1, taken largely from Davis et al. (1970) compares the characteristics of a number of other similar hydrogen bacteria with the isolate. As can be seen, the isolate appears to be identical to *Alcaligenes paradoxus*.

In addition, the isolate possessed "degenerately peritrichous" flagella, generally one or two, which originated subpolarly and extended several times the length of the cell, and a characteristically unpleasant odor when grown autotrophically. Both of these characters also agreed well with the description of *A. paradoxus*.

The properties for which there is no comparable information regarding *A. paradoxus* are: negative for indole formation, nitrate is reduced to nitrite, sulfide is not produced, a heavy pellicle is formed in SIM medium, and no growth occurs with L-(+)-cysteine.

Growth under autotrophic conditions resulted in such a tenacious slime that it was extremely difficult to obtain individual colonies by ordinary streaking. On the other hand, growth on TSA plates resulted in soft colonies that were easy to pick and restreak, resulting in good colonial separation.

Triplicated dry weight determinations from autotrophically grown cells yielded 1.67×10^{-13} g/cell with a standard deviation of 7.51×10^{-15} g/cell.

SECTION 2. GROWTH STUDIES

INTRODUCTION

Due to their unique dual modes of growth, the hydrogen bacteria have been the subject of numerous growth studies involving either their autotrophic, heterotrophic, or mixotrophic (autotrophic and heterotrophic processes occurring simultaneously) metabolism. For example, studies have been done on the possible use of certain hydrogenomonads in closed cycle systems

TABLE 1. A COMPARISON OF SOME CHARACTERISTICS OF OTHER GRAM-NEGATIVE, ROD-SHAPED, YELLOW-PIGMENTED HYDROGEN BACTERIA WITH THE TRITIUM-OXIDIZING ISOLATE (TAKEN LARGELY FROM DAVIS ET AL., 1970)

	Pseudomonas flava	Strain 450	*Pseudomonas palleroni*	Strain 363	*Alcaligenes paradoxus*	Unknown Isolate
Yellow Pigmentation	+	+	+	+	+	+
Oxidase Reaction	+	+	+	+	+	+
Catalase Reaction	+	+	+	+	+	+
Motility	+	+	+	-	+	+
Tolerance to 20% O_2 with H_2	-	+	±[b]	+	(+)[a]	+
Hydrolysis of Starch	-	-	-	-	-	-
Organotrophic Denitrification	-	-	-	-	-	-
Utilization of:						
H_2	+	+	+	+	+	+
glucose	+	+	+	+	+	+
D-xylose	-	(+)[d]	-	-	+	+
L-arabinose	+	+	-	-	+	+
D-mannose	+	+	-	-	+	+
D-galactose	+	+	-	-	+	+
sucrose	+	+	-	-	-	-
mannitol	+	+	-	-	+	+
para-hydroxybenzoate	-	-	+	-	+	+
L-leucine	-	+	+	+	+	+
L-tryptophan	-	-	+	-	±[d]	+
histidine	-	+	-	-	±[c]	+

[a] Parentheses indicate that mutation and selection may be required before the indicated reaction occurs.

[b] One of two strains is positive, the other negative for this character.

[c] Ten of eleven strains are known to be positive for this character.

[d] Seven of eleven strains are known to be positive for this character.

for waste recycling and protein production in spacecraft (Foster and Litchfield, 1964; 1969) and other continuous culture systems (Amman et al., 1968; Bongers, 1970). Many studies have also been done determining the nutritional and gaseous requirements for autotrophic growth (Schatz and Bovell, 1952; Repaske, 1962; Bartha and Ordal, 1965; Amman and Reed, 1967; Repaske et al., 1971; Repaske and Repaske, 1976; Repaske and Mayer, 1976). All these investigations were concerned with autotrophic growth and the rates of several species were established.

In a similar manner growth studies involving the metabolism of various organic compounds including amino acids (Fraser-Smith et al., 1968; Brown and Clark, 1974; DeCicco and Stukus, 1968) and carbohydrates and/or organic acids (Rittenberg and Goodman, 1969; Kluyver and Manten, 1942; Wilson et al., 1953; Crouch and Ramsey, 1962; Cook et al., 1967; Marino and Clifton, 1955; Stukus and DeCicco, 1970) have been done. Most of these studies on heterotrophic growth were also concerned with mixotrophic growth, and from these it was generally found that addition of an organic compound to autotrophically growing cells resulted in increased gas uptake, rate of growth, and cell yield. Frequently, there was a lag period between the time of addition of the organic substrate and the subsequent increased metabolic activity. This lag apparently represented the time required to synthesize the enzymes necessary for the metabolism of the added substrate.

The object of this study was to determine the growth rate of *A. paradoxus* in a suitable mineral salts medium under an appropriate gas mixture of hydrogen, oxygen, and carbon dioxide. This was done at different pH's to establish the range of tolerance under autotrophic conditions. Next, after an optimal pH had been established, various organic nutrients were added to the media to evaluate their effect on the growth rate.

MATERIALS AND METHODS

Initially, *A. paradoxus* was grown in the autotrophic media used for the dry weight determinations; however, pH control was very difficult due to the use of NH_4Cl. Therefore, Repaske's media were modified by replacing the NH_4Cl with 10^{-2} M $(NH_4)_2HPO_4$. This also eliminated the need for a separate phosphate addition. Thus, the modified growth media contained the following reagents in the listed final concentrations:

$(NH_4)_2HPO_4$	1.0×10^{-2} M
$MgSO_4 \cdot 7H_2O$	5.0×10^{-4} M
$CaCl_2 \cdot 2H_2O$	7.0×10^{-5} M
$NaHCO_3$	1.2×10^{-2} M
$Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$	6.0×10^{-5} M

One percent of the trace metals stock solution described by Repaske (1962) was also added. The iron was sterilized by filtration through 0.22-micrometer (μm) Millex filters (Millipore) and added after the other ingredient had been

autoclaved. Distilled water was always used and the pH was adjusted with HCl or NaOH. The pH was checked after autoclaving and several times thereafter during the growth rate determinations.

Cultures were grown in 1-liter volumetric flasks with sidearms suitable for insertion into a Bausch & Lomb Spectronic 20 colorimeter. Each flask contained 250 ml of media which was then inoculated with 1 ml of a fully grown fresh culture of *A. paradoxus*. Since a fully grown culture of *A. paradoxus* contains about 2 to 5 x 10^9 cells/ml, this inoculum resulted in an initial cell concentration of approximately 10^7 cells/ml. Growth of the culture and thus estimation of the cell concentration was possible using the colorimeter. This facilitated finding the proper dilution necessary for accurately determining the cell numbers at the sample times. Dichromate blanks with the same optical density as the uninoculated media were prepared for each flask. Thereafter, the appropriate blank was used to zero the colorimeter before a reading was taken. A wavelength of 540 nm was used (DeCicco and Stukus, 1968) for all readings. In this manner it was found that the correct dilution factor could consistently be selected.

A gas line was connected to each flask via a glass tube through each flask's neoprene stopper. The gas pressure was kept near 1 atmosphere by using two 10-liter carboys with a water displacement system as described in Repaske (1962). The flasks were shaken in a water bath maintained at 30° C.

Samples were removed from each flask through a septum on the end of a short length of glass tubing that penetrated the stopper. Pre-sterilized, disposable 1-cubic centimeter (cm^3) syringes were used. It was found that somewhat more variation than was desired occurred while attempting to remove exactly 1.0 ml of the culture for dilution purposes. Therefore, in the first dilution only 0.1 ml of the culture was used since it was possible to obtain much better accuracy when going from mark-to-mark on the syringe as opposed to expelling the entire contents.

Dilutions were done in blanks filled with tap water. Since accurate knowledge of the dilution factors was of prime importance the dry dilution bottles and their caps were individually weighed, water was added, and then the bottles were steam sterilized. After sterilization, they were allowed to cool, all external moisture was allowed to evaporate, and they were then reweighed. The difference in weights (in grams) was considered to be exactly equal to the number of milliliters of water in each blank. Using this technique, consistently accurate cell counts were obtained.

Cell counts were done using spread plates of TSA layered with from 0.1 to 0.4 ml of diluent. Four plates were prepared for each sample.

For growth studies involving the addition of organics to the autotrophic media, three of the organics used, dextrose, fructose, and leucine, were added by filtration to the autoclaved mineral salts media. These organic additions were brought up to a final concentration of 10^{-2}M. The fourth organic used was trypticase soy broth (TSB) (BBL) which was too viscous to be filtered and so was separately autoclaved and then added to a final concentration of one-half its normal strength, i.e., 3.25 g/250 ml.

The heterotrophic/autotrophic studies with the organics were run in two different ways. In the first, the organics were added to the mineral salts medium at the same time that the cells were added. In the second, the inoculum was added and the cells were allowed to undergo one or two divisions autotrophically before the addition of the organics.

Aseptic technique was always observed when working with sterile materials, and samples were withdrawn from flasks as quickly as possible to avoid excessive interruption of growth. Also, in experiments where organics were added to the growth media, the gas mixture in the carboys was frequently changed even though more than half of it had not yet been utilized. This was done because of possible radical changes in the ratios of the gases due to simultaneous growth under both autotrophic and heterotrophic conditions.

RESULTS AND DISCUSSION

There were two ways of determining the growth rate of *A. paradoxus*. The exponential growth rate constant, k, could have been determined by the following equation (Stanier et al., 1966):

$$k = \frac{\log_2 N_t - \log_2 N_0}{t}$$

where

N_t = cell concentration at time t

N_0 = cell concentration at time zero

t = time in hours.

Thus, k represents the number of generations per hour. The reciprocal of this, 1/k, could also be computed. This represents the mean doubling time and is in hours per generation.

The other way of determining the growth rate, if during exponential growth the cell concentration at more than two times is known, is to plot the $\log_2$ of the cell concentration versus the time in hours and determine the slope of the regression line that best fits the points. This can be done because during exponential growth such a semilogarithmic plot should yield a straight line (Stainer et al, 1966). The slope thus determined is equal to k. Also, every unit increase on the $\log_2$ scale represents one doubling of the cells.

This latter method was used for the determination of both k and 1/k in all growth experiments shown except for the autotrophic growth of *A. paradoxus* at pH 5.0 (Table 2) in which usable data points were obtained for only two times. In all other cases data points at three or more times were obtained and so the regression line was used. In these cases the points were statistically examined to determine the validity and usefulness of the data. A lack of fit test was run to determine if there was a significant lack of fit of the points to a straight line at either the 95 percent or 99 percent confidence

levels. A regression test was also run on each set of points to determine, at the same confidence levels, if there was a significant regression.

The results of the autotrophic growth experiment determining the range of pH tolerance are shown in Figure 1 and Table 2. No growth occurred at pH 4.0 or 10.0.

TABLE 2. A COMPARISON OF THE AUTOTROPHIC GROWTH RATES OF *Alcaligenes paradoxus* AT DIFFERENT pH's

pH	k (generations/h)	1/k (h/generation)
5.0	0.146	6.480
6.0	0.165	6.059
7.0	0.156	6.402
8.0	0.105	9.485
9.0	0.086	11.662

For the slow rate of autotrophic growth it is more meaningful to refer to the mean doubling time when comparing the rates at different pH's. This way it can be seen that the shortest doubling times occurred at pH 6.0 followed closely by pH 7.0 and 5.0. The others, pH 8.0 and 9.0, were considerably slower. This range for autotrophic growth is not surprising based on the findings of Lascelles and Still (1946) who determined the pH optimum for *E. coli* was 5.9 to 6.4, Schatz and Bovell (1952) who found the highest rate of oxidation for another hydrogenomonad, *Hydrogenomonas facilis*, to be at pH 6.0, and Repaske (1962) who obtained the highest rate of growth of *Hydrogenomonas eutropha (Alcaligenes eutrophus)* at pH 6.6.

For growth involving organic additions, two sets of experiments were run. In the first, the cells were added to the flask at the same time as the various organics. In the second, the cells were added first and the organics added after one or more autotrophic divisions had occurred. In this way the lag period between strictly autotrophic growth and heterotrophic/autotrophic growth could be better determined. Both sets of experiments had a control flask consisting of the autotrophic medium at pH 7.0.

The results of the first run are shown in Table 3. It can be seen that TSB gave the shortest doubling times followed by fructose, leucine, glucose, and the pH 7.0 control, in that order. Statistically, none showed a significant lack of fit to a straight line and the regression was significant at the 99 percent confidence level.

In the second run, the organics were added after the cultures had been maintained for 16 hours under autotrophic conditions. It was found that with the glucose and fructose additions a statistically significant increase in slope (growth rate) occurred within 2 hours or less (Figures 2 and 3).

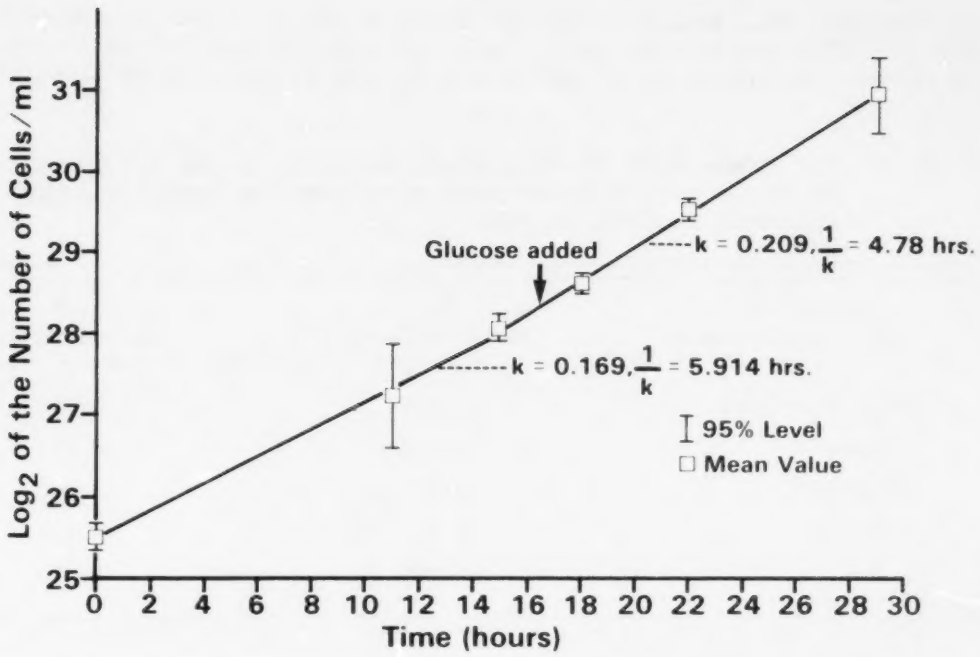

Figure 1. The autotrophic growth rate, k (generations/hour), of *Alcaligenes paradoxus* as a function of the pH.

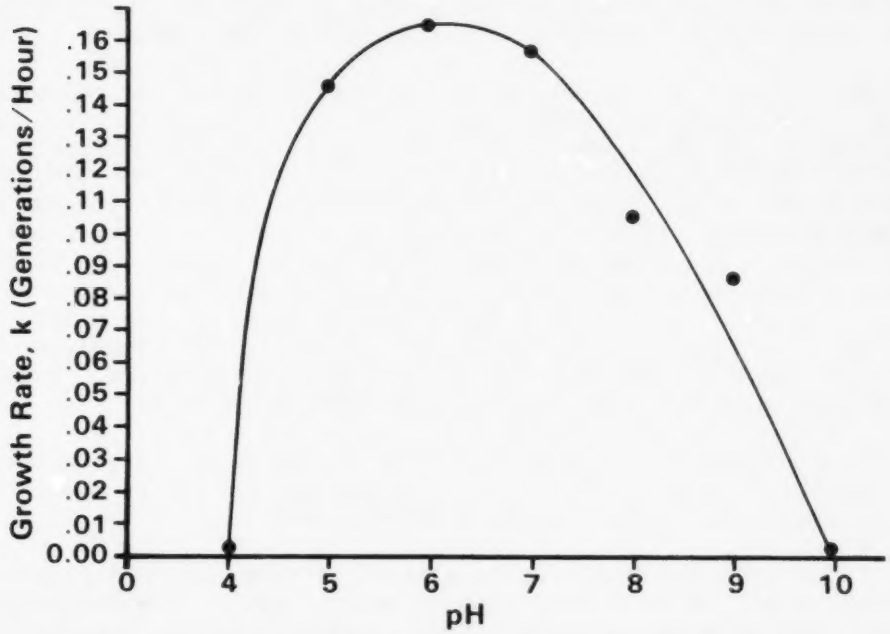

Figure 2. The growth rates of *Alcaligenes paradoxus* in the autotrophic medium before and after the addition of glucose.

In addition, the lines described by both the 0-, 11-, and 15-hour points and the 18-, 22-, and 29-hour points each had a significant regression at the 99 percent confidence level and showed no significant lack of linearity.

TABLE 3. THE GROWTH RATE OF *ALCALIGENES PARADOXUS* IN THE AUTOTROPHIC MEDIUM AT pH 7.0 (CONTROL) AND WITH SUPPLEMENTS OF EITHER GLUCOSE, FRUCTOSE, LEUCINE, OR TSB

Supplement to autotrophic media	k (generations/h)	1/k (h/generation)
None (control)	0.174	5.738
Glucose	0.219	4.570
Fructose	0.326	3.066
Leucine	0.229	4.364
TSB	0.565	1.770

For some reason the addition of leucine, Figure 4, actually decreased the growth rate even though it had been used previously with success both in liquid and on solid media. No explanation is available for this result. Further tests would be needed to help clarify this response.

As for the TSB, it had four separate rates of growth, as can be seen in Figure 5. The rate of growth between 0 and 11 hours is the true autotrophic growth rate since the decrease between 11 and 18 hours can be attributed to the half hour during that time that the flask was not being shaken or connected to its gas supply while some problems in adding the TSB to the flask were resolved. Therefore, the doubling time of 5.030 hours was considered correct. After the addition of the TSB, the growth rate greatly increased between 18 and 22 hours, then decreased somewhat between 22 and 29 hours. The decrease was probably due to depletion of some nutrient or overcrowding since at 22 hours the cell concentration had already exceeded 10^9 cells/ml which is very near the maximum generally obtained. Thus, the doubling time after the addition of TSB was considered to be 2.398 hours.

The control flask, Figure 6, showed no variation in slope during the entire experiment, and a statistically significant regression and fit of a single straight line through all data points could be obtained.

In Table 4 the different doubling times obtained during the period of autotrophic growth in this second set of organic additions experiments are compared. The range was from about 5 to 6 hours. Doubling times found by other investigators for other hydrogen bacteria are: 3.2 hours for *Hydrogenomonas* H 20 (Schlegel, 1966), 1.7 hours with *Hydrogenomonas eutropha* (Bongers, 1970), 3.5 to 4.0 hours with *Hydrogenomonas eutropha* (Repaske, 1962),

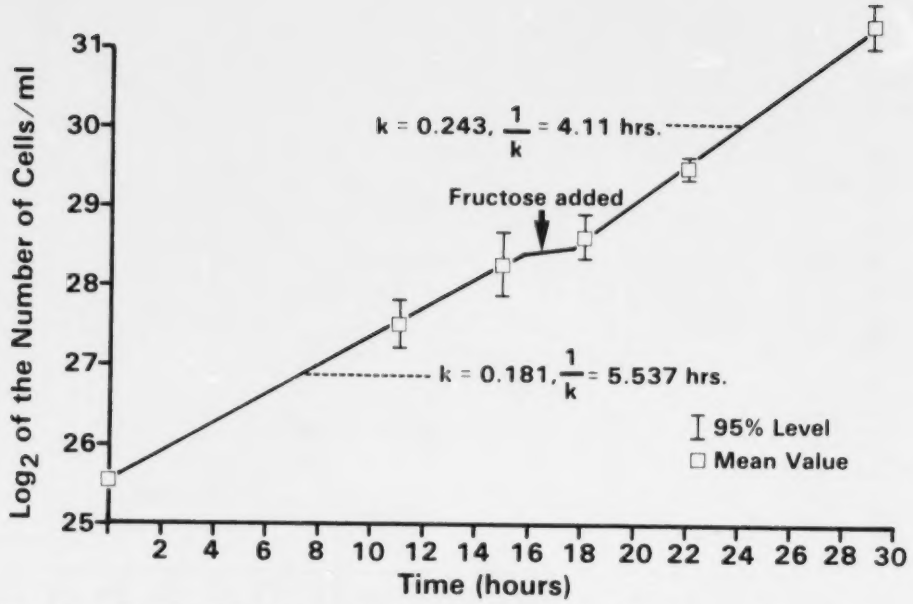

Figure 3. The growth rates of *Alcaligenes paradoxus* in the autotrophic medium before and after the addition of fructose.

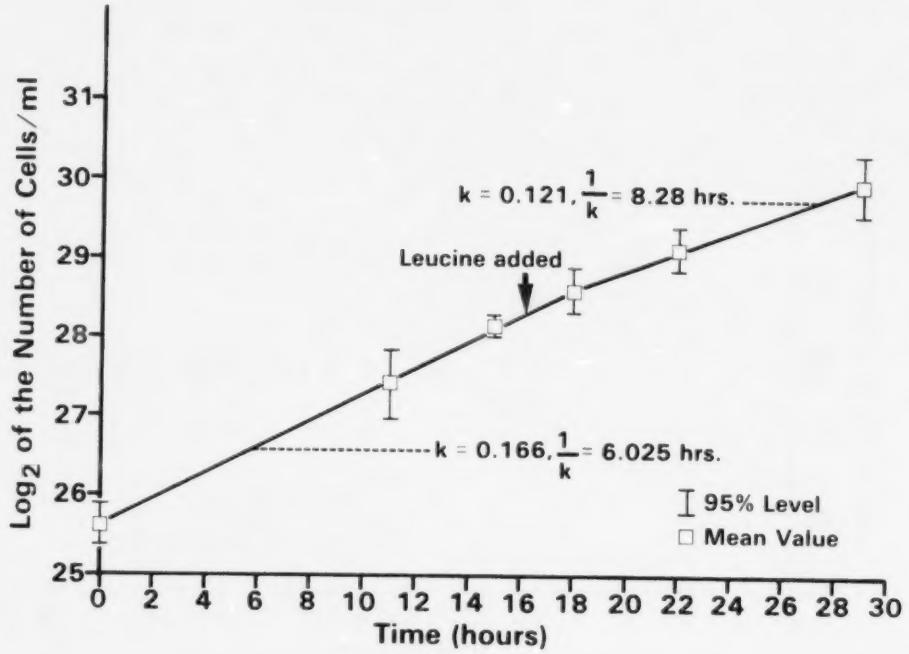

Figure 4. The growth rates of *Alcaligenes paradoxus* in the autotrophic medium before and after the addition of leucine.

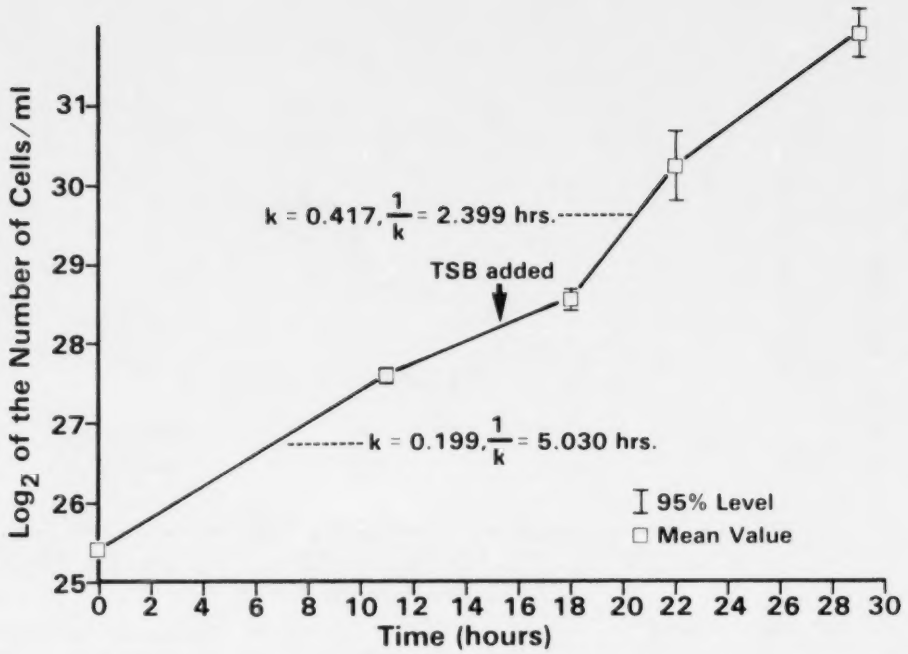

Figure 5. The growth rates of *Alcaligenes paradoxus* in the autotrophic medium before and after the addition of TSB.

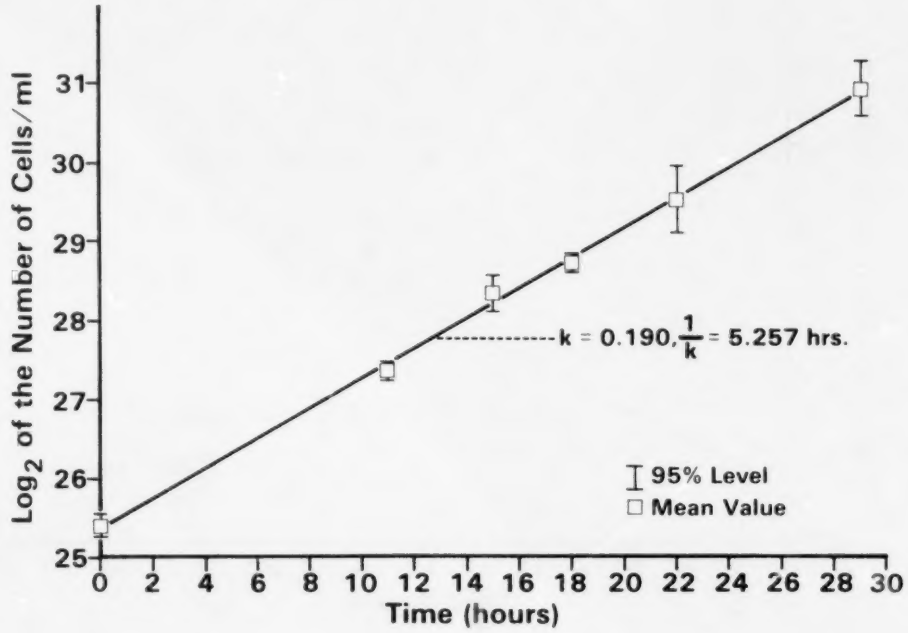

Figure 6. The growth rate of *Alcaligenes paradoxus* in the autotrophic medium at pH 7.0.

and 2 hours with *Alcaligenes eutrophus (Hydrogenomonas eutropha)* (Repaske, 1966). No growth experiments using *A. paradoxus* have yet been published.

TABLE 4. AUTOTROPHIC DOUBLING TIMES OF *Alcaligenes paradoxus* (HOURS)

pH 7.0 control		5.257
Leucine flask	(before organics added; 0 to 15 h)	6.025
Glucose flask	(before organics added; 0 to 15 h)	5.914
Fructose flask	(before organics added; 0 to 15 h)	5.537
TSB flask	(before organics added; 0 to 11 h)	5.030
	$\bar{x}$ =	5.553
	standard deviation =	0.423

The autotrophic doubling times obtained in these experiments are somewhat longer due to the following critical factors that have been shown to be important (Repaske, 1962, 1966; Schlegel, 1966):

1) good aeration
2) supplements of nutrients as needed
3) pH control
4) proper gas mixture

Of these, the first is the only factor which really affected these results. For the period of time growth was followed, there was no nutrient depletion or pH fluctuation, and in addition the gas supply was frequently changed. However, adequate aeration was a problem since the shaking water bath used was not capable of high speeds and the flasks used were smooth-walled. Baffles on the inside walls would have greatly increased aeration, however, such flasks were not available in time to be used. Also, some of the doubling times of other investigators, namely the 1.7- and 2-hour times, were obtained using continuous culture apparatus with high impellor speeds and electrodes to constantly monitor the dissolved gas concentrations and pH. In this manner they were able to maintain exponential growth almost indefinitely.

In the experiments involving the organic additions, a question arises regarding the mechanisms responsible for the increase in growth after the addition of the organics (the second run with leucine excepted). There exist two possibilities:

1) Heterotrophic growth occurred after the addition of the organics and autotrophic growth ceased;

2) Both autotrophic and heterotrophic growth occurred simultaneously (mixotrophic growth).

Mixotrophic growth of the closely related *Alcaligenes eutrophus* has been shown by DeCicco and Stukus (1968), Rittenberg and Goodman (1969), Stukus and DeCicco (1970), and Brown and Clark (1974). *Hydrogenomonas (Pseudomonas) facilis* has also been shown to exhibit mixotrophic growth (Wilson et al., 1953; Brown and Clark, 1974; DeCicco and Stukus, 1968). Thus, it would not be surprising if *A. paradoxus* was also capable of it.

Although determination with certainty of mixotrophic growth would be useful, it was not the intention here. Rather, this study has shown the growth rate under autotrophic and autotrophic/heterotrophic conditions.

SECTION 3. RESTING CELL STUDIES

INTRODUCTION

In many instances an experiment may require that the number of bacterial cells be constant while measuring some parameter. Bacteria can be prepared in this way by suspending a known number of cells in some medium that will not greatly affect their reactivity or viability and will also not allow cell division to occur. Such suspensions are frequently known as "resting cells" although they are certainly not "resting" except from cell division.

Many investigators have used this technique to observe the uptake of gases by hydrogenomonads (Kluyver and Manten, 1942; Marino and Clifton, 1955; Schatz and Bovell, 1952; Schatz, 1952; Lascelles and Still, 1946; Packer and Vishniac, 1955; Atkinson, 1955; Lindsay and Syrett, 1958; Kanai et al., 1960) under autotrophic, heterotrophic and mixotrophic conditions.

In this study suspensions of the soil isolate, *A. paradoxus*, were prepared in buffers of varying pH to determine the range of their tritium-oxidizing ability. The effect of organics on this reaction is also examined.

MATERIALS AND METHODS

Cultures of *A. paradoxus* were prepared under strict autotrophic conditions as described in the preceding chapter. These cells were then centrifuged down at 4,000 to 5,000 x g for at least 20 minutes. The supernate was decanted, and the cells were rinsed twice with distilled water, then resuspended in a small volume of the same.

pH Study

One-liter, round-bottomed flasks were used. A series of 8 or 9 flasks was used for each pH examined. Each of the 8 or 9 flasks had 15.0 ml of 0.025M potassium phosphate buffer at the desired pH and the flasks were then positioned on a rotary shaker. Next, approximately 0.5 ml of the washed cell suspension was added to each flask using an automatic pipette. The pipette had previously been checked for accuracy and was found to consistently deliver the same volume within 0.001 ml. Thus, each flask could be expected to start with the same cell concentration. This was verified

by cell counts on TSA spread plates using appropriate dilutions. During the entire inoculation time the flasks remained unstoppered.

When this was complete, each flask was stoppered and injected with 5 cm^3 of nitrogen gas containing 930 nanocuries (nCi) of elemental tritium ($^3H_2$). The stoppering and injecting took place together and the exact time of injection of each individual flask was recorded.

When all the flasks were injected (which usually took only 10 to 15 minutes), the shaker was started. Flasks were then removed at the desired times and flushed with air which removed any remaining tritium, thus stopping the reaction, and the contents were poured into a small vial and capped.

After all the samples had been collected, they were filtered through 0.045-μm filters which removed the cells and any other debris. The filtrate could then be counted by liquid scintillation as described by Lieberman and Moghissi (1970). Appropriate quenching standards were prepared to correct for the presence of the buffer. In this manner, the precise amount of gaseous tritium converted to tritiated water could be determined for each sample.

This produced a sequence of points which yielded a curve described by a regression function known as the exponential growth model:

$$Y = P_1[1-EXP(-P_2t)] + E \qquad (1)$$

where

Y = tritium level
P_1 = the asymptotic tritium concentration
P_2 = the reaction rate parameter
t = the time in hours
E = the error function, assumed to be Gaussian

The derivative of formula (1) with respect to time gives the velocity of the reaction.

$$\frac{dy}{dt} = P_1P_2e^{-P_2t} \qquad (2)$$

The velocity is maximal and equals P_1P_2 at time zero. In this manner the initial velocity (calculated at time zero) and asymptote were determined for each pH examined.

Organic Effect Study

The cells were prepared as before. However, all the flasks except those using TSB had 15.0 ml of the buffer at pH 7.0 with a 10^{-2}M concentration of either glucose, fructose, leucine, or histidine. The series with TSB as the

organic source used only the broth prepared at its normal strength (7.5 g/ 250 ml). Replicate series of pH 7.0 were also run as a standard against which to compare the rate of oxidation in the presence of the organics.

Due to the presence of the organics, it was decided to autoclave all the flasks and materials that came into contact with either the cells or media. Cell counts were done as previously described. The injection of tritium was performed just as before and the shaker started.

Collection of the sample after the specified time was done somewhat differently. The reaction was stopped as before by opening the flask and flushing it with a stream of air, but then the water was extracted in a benzene azeotrope (Moghissi et al., 1973). As before, appropriate standards were prepared and the tritium content was determined by liquid scintillation. Mathematical computations of the initial velocity and the asymptote of the reactions were done as in the pH study.

RESULTS AND DISCUSSION

The results obtained from the resting cell experiments provided the desired preliminary data. Before pursuing further serious investigation into the potential of this organism for use as a biological monitor, it was necessary to determine what effect two important environmental factors, pH and organics, would have on the ability of *A. paradoxus* to oxidize tritium. No attempt was made to accurately determine the variation from pH-to-pH or organic-to-organic. Rather, it was important only to know if the oxidation reaction occurred at all or was very severely inhibited or enhanced under the various conditions examined. The results obtained answered these questions.

No conversion of tritium to tritiated water was found to occur at pH 3.0 or 10.0. The rates and limits of the reactions from pH 4.0 to 9.0 are shown in Table 5. As can be seen, the initial velocities and asymptotes for the oxidation reaction at these pH's were quite similar. This would seem to indicate that tritium oxidation occurs at a near optimal rate over a wide range of hydrogen ion concentrations. This may prove to be quite important since, as will become evident in the next section, the intent was to develop this organism as a biological monitor; and in environmental samples, whether they be soil or water, a wide range of pH's can be expected.

The effect of the addition of organics can be seen in Table 6. It should be noticed that the initial velocities for the reactions at pH 7.0 are all much slower here than those obtained in the pH study. This can be attributed to batch-to-batch variation in the hydrogen dehydrogenase activity of cell cultures. However, within any one batch of cells the activity was constant.

The addition of organics was found to cause no shutdown of the tritium-oxidizing activity. It appears from Table 6 that, if anything, some of the organics actually caused a slight increase in the reaction rate. However,

no significance can be given to that possibility since this experiment was run only once and one would want to run at least triplicates to be fairly sure of the variation around each data point.

TABLE 5. THE RATE AND EXTENT OF TRITIUM OXIDATION BY RESTING CELLS OF *Alcaligenes paradoxus* AT DIFFERENT pH's

pH	initial velocity (nCi/h)	asymptote (nCi)
4.0	175	706
5.0	141	800
6.0	151	785
7.0	139	857
8.0	172	788
9.0	123	767

TABLE 6. THE RATE AND EXTENT OF TRITIUM OXIDATION BY RESTING CELLS OF *Alcaligenes paradoxus* IN THE PRESENCE OF DIFFERENT ORGANICS

sample	initial velocity (nCi/h)	asymptote (nCi)
pH 7.0 #1	46	628
pH 7.0 #2	42	736
Glucose	47	821
Fructose	53	706
Leucine	55	668
Histidine	62	638
TSB	41	671

These data clearly show that the addition of *A. paradoxus* to an environmental sample with high organic levels will not result in inactivation of the oxidation reaction. While it is certainly true that not all possible organics one could expect to find in nature were examined, the TSB series showed good activity; and TSB contains a number of protein digests and yeast extract, and so has a fairly high concentration and variety of organic compounds. And certainly, a 10^{-2}M concentration of the other organics tested is far higher than one would expect in almost any environmental sample.

SECTION 4. HEAVY METAL EFFECT STUDIES

INTRODUCTION

Numerous studies have been done on the toxicity of mercury to animals, plants, man, and microorganisms. Similar investigations have been conducted into the toxicity of cadmium and lead. A number of excellent reviews on mercury (Friberg and Vostal, 1972), cadmium (Friberg et al., 1971), and lead (U.S. Public Health Service, 1966) are available in addition to many less comprehensive publications on the toxicity, availability, removal of these metals, and the general reactions they can undergo.

A common problem encountered by these investigators is the determination of the actual biologically available levels of these compounds in the sample being examined. The absolute concentration can be determined by a number of methods, but these concentrations may not necessarily correspond to the levels free to react with a biological system. In general, it has been found that organic compounds in particular will strongly bind with mercury, lead, and cadmium in this order of affinity:

$$Hg^{2+} > Pb^{2+} >> Cd^{2+} \qquad (3)$$

(Ramamoorthy and Kushner, 1975). Also, clay soils have been shown to reduce the toxicity of cadmium (Babich and Stortzky, 1977b) and mercury (van Fraassen, 1973) compared to sand soils. The pH effect on cadmium toxicity has also been examined with regard to various bacteria, fungi, and actinomycetes (Babich and Stortzky, 1977a).

The object of this study was to examine the effect of mercury, lead, and cadmium on the rate of oxidation of tritium (hydrogen) by the soil isolate of *A. paradoxus* previously described. The hope was to use the techniques acquired from the resting cell studies to analyze rapidly samples for the bioavailable concentration of the particular metal of interest as opposed to current methodology which can analyze only for levels chemically available. This study also examines the effect in soil of these metals on the rate of tritium oxidation.

MATERIALS AND METHODS

Cells of *A. paradoxus* were cultured, washed and resuspended as described in the previous Section.

Mercury, Cadmium, and Lead in Solution Experiments

One-liter, round-bottomed flasks were prepared with 0.025M potassium phosphate buffer, pH 5.0, 7.0, or 7.2 as detailed in the previous Section. These contents were then amended with either $Hg(NO_3)_2$, $CdCl_2$, or $PbCl_2$ to yield the desired final concentrations. Controls received a volume of distilled water equal to the volume of the metal solution addition. Cells were then added to the flasks which were stoppered, injected, and shaken as

described before. The final volume of liquid within each flask was 15.0 ml. Collection of the water by benzene distillation and the analysis for its tritium content were also done as before.

Mercury-in-Soil Experiments

Twenty grams of steam-sterilized clay loam were put into each one-liter, round-bottomed flask. Sterilization was verified by plating soil dilutions on TSA. After this, 14.0 ml of the *A. paradoxus* cell suspension and 1.0 ml of the proper dilution of $Hg(NO_3)_2$ were added to yield a final concentration of 1.0, 10.0, or 100.0 ppm. Controls received 1.0 ml of distilled water instead of the mercury solution. The flasks were then stoppered and injected and the mixture was well shaken to create a "slurry" covering the entire inner surface of the flask. The flasks were then incubated without shaking in a room at 30° C. Samples were removed and the water distilled and analyzed as before.

Cadmium-in-Soil Experiments

Field soils were obtained which had been amended with cadmium (as $CdCl_2$) in 1973. In the years since then the levels of residual cadmium have steadily decreased as shown in Table 7 (A. G. Wollum, personal communication). The 1977 data were obtained for this study by extraction of the soils using the methods of Follett and Lindsay (1971) followed by atomic absorption analysis.

At the time of this study the levels of cadmium in all four soils were near background concentrations. The pH's of the soils were also very similar being 5.1, 4.8, 5.2, and 4.8, respectively.

In the experiments, 20 g of the soil was put into each flask followed by the addition of a sufficient volume of the cell suspension to obtain a good slurry of the mixture on the inside of the flasks. The injection, stoppering, and analysis were done as before.

TABLE 7. THE CONCENTRATION (IN ppm) OF CADMIUM (AS $CdCl_2$) MEASURED IN THE FIELD SOILS AMENDED WITH 0 (SOIL 1), 3 (SOIL 2), 11 (SOIL 3), AND 20 (SOII 4) ppm CADMIUM IN 1973

Year	Soil 1	Soil 2	Soil 3	Soil 4
1974	0.20	2.16	5.58	12.37
1975	0.19	0.86	2.35	4.03
1977	0.18	0.24	0.26	0.28

Cadmium and Lead Effect on Cells in a Solution of EDTA and Tris

Each flask was prepared as in the lead and cadmium solution experiments. The buffer was always pH 7.0; however, the washed cells, diluted to a concentration of approximately 5 X 10^4 g/ml were suspended in 30 mM tris-(hydroxymethyl)-aminomethane (Tris), pH 8.0, and 10^{-2}M disodium ethylenediaminetetraacetic acid (EDTA) (Repaske, 1958) for either 40 minutes at room temperature or 48 hours at 7° C, and then recentrifuged and washed twice more in distilled water to remove the Tris and EDTA. The cells were next pipetted into the test flasks which were then stoppered, injected with the tritium, and shaken for the desired time. Removal, extraction of the water, and tritium analysis were done by benzene distillation and liquid scintillation as previously described.

RESULTS AND DISCUSSION

Mercury Studies

The results of a variety of mercury concentrations in solution, pH 7.2, on the ability of *A. paradoxus* to oxidize tritium are shown in Figure 7. It is evident that *A. paradoxus* is very sensitive to somewhere between 0.1 and 1.0 ppm mercury in an uncomplexed solution. In the sterilized soil, Figure 8, amended with *A. paradoxus* and mercury, 1.0 ppm was sufficient to completely inhibit the oxidation reaction, but only after approximately 20 percent conversion of the tritium to tritiated water had taken place. For the same concentration of mercury in solution, only about 5 percent conversion took place. It is noteworthy that 100.0 ppm mercury in the soil produced the same effect as 1.0 ppm in solution. This suggests that the clay loam soil offered some degree of protection not available in solution. This is consistent with the results obtained by Babich and Stortzky (1976b) who found that cadmium toxicity was reduced by clay minerals. They determined that the degree of protection was directly related to the cation exchange capacity of the clays used. Work by van Faassen (1973) seems to indicate that this is true for mercury also since he found that in sandy soils the toxicity of mercury was greater than in clay soils. Thus, since the soil used here was a clay loam, it should not be surprising that some protection from mercury was afforded to the cells. The negatively charged clay particles could be binding the Hg^{2+} ions, thus preventing their interaction with the cells. Exchange of the Hg^{2+} ions with other cations in the solution probably accounted for the eventual toxic effect on the bacteria.

A concern in determining the usefulness of *A. paradoxus* for future biological monitoring was that it might be resistant to low levels of heavy metals. For mercury this appears not to be the case. Although this study is far from conclusive, it clearly shows that *A. paradoxus* is sensitive to low levels of mercury both in solution and soil. Future work could fill in the gaps and cover a larger range of mercury concentrations and conditions of exposure. It seems that *A. paradoxus* has the potential, given sufficient research emphasis, of serving as a sensitive bioindicator of environmental mercury.

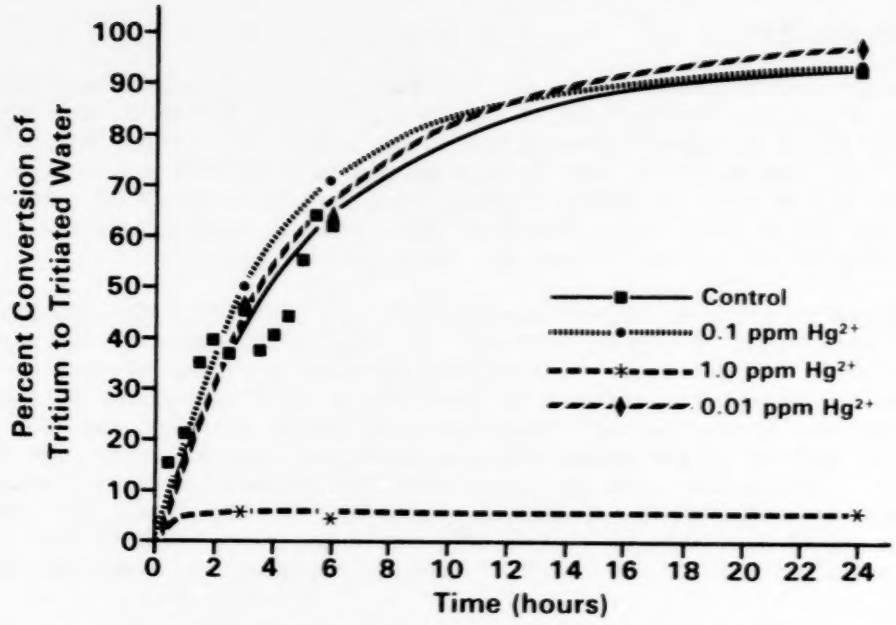

Figure 7. Tritium oxidation by *Alcaligenes paradoxus* in 0.025M potassium phosphate solution, pH 7.2, and amended with either 0.01, 0.1, or 1.0 ppm mercury [as $Hg(NO_3)_2$].

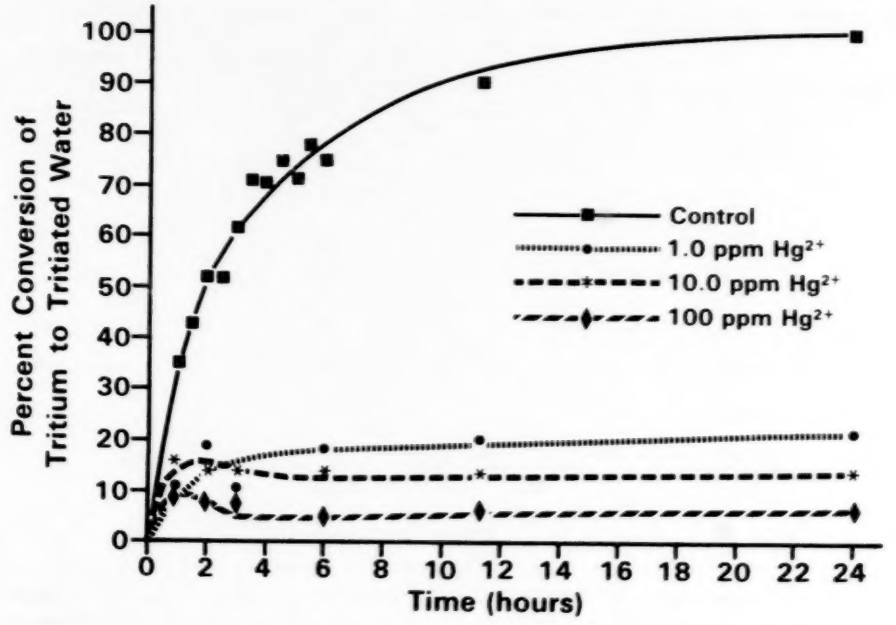

Figure 8. Tritium oxidation by *Alcaligenes paradoxus* in sterilized clay loam soil, and amended with either 1.0, 10.0, or 100.0 ppm mercury [as $Hg(NO_3)_2$].

Cadmium and Lead

The results obtained from the incubation of *A. paradoxus* with low levels of cadmium and lead at pH 7.2 in a phosphate buffered solution are shown in Table 8. They reveal a different pattern than found for mercury since here even 10.0 ppm lead or cadmium had no discernible effect on the ability of the cells to oxidize the tritium to tritiated water. Therefore, a similar test, this time using 100.0 ppm lead and cadmium was run; however, once again no inhibitory effect was noted (Figure 9).

Since to use even higher concentrations to establish the upper limit of sensitivity would have been pointless in light of the planned use for *A. paradoxus* as a monitor of low environmental levels of the metals, it was decided to attempt to increase the sensitivity of *A. paradoxus* to the lead and cadmium. Thus, the cells were again incubated with 1.0 and 10.0 ppm of lead and cadmium in phosphate buffered solution, however, this time the pH was 5.0. The results were identical to those before, namely no reduction in the rate or extent of the oxidation reaction. At this pH all the lead and cadmium could certainly be expected to be in solution (as Pb^{2+} and Cd^{2+} ions), so it appeared that *A. paradoxus* was fairly resistant to these two metals.

TABLE 8. THE RATE AND EXTENT OF TRITIUM OXIDATION BY RESTING CELLS OF *Alcaligenes paradoxus* IN A SOLUTION OF 0.025M POTASSIUM PHOSPHATE, pH 7.2, AMENDED WITH LEAD (AS $PbCl_2$) OR CADMIUM (AS $CdCl_2$)

Concentration (ppm) and Element	Initial Velocity (% conversion/h)	Asymptote (% conversion)
Control	15	95
0.1 Cd^{2+}	13	99
1.0 Cd^{2+}	14	92
10.0 Cd^{2+}	13	98
0.1 Pb^{2+}	17	91
1.0 Pb^{2+}	14	97
10.0 Pb^{2+}	13	90

It was then found that the effect of certain antibiotics could be enhanced, presumably by increasing bacterial permeability to them (Weiser et al., 1969; Brown and Richards, 1965; Heppel, 1967). These techniques all required pretreatment of the bacterial cells with the disodium salt of EDTA followed by the antibiotic exposure.

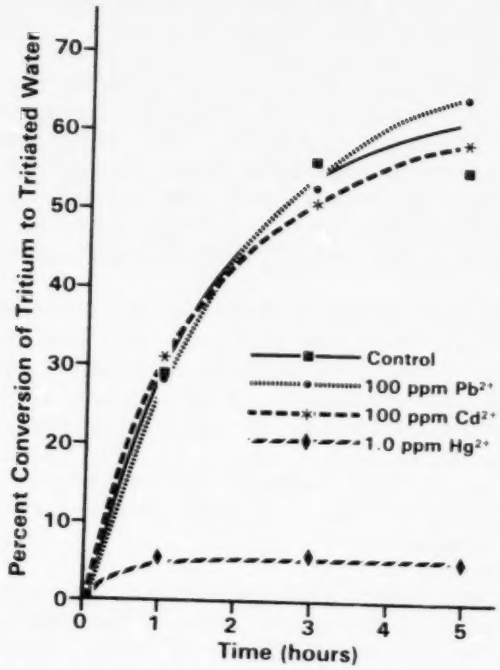

Figure 9. Tritium oxidation by *Alcaligenes paradoxus* in 0.025M potassium phosphate solution, pH 7.2, and amended with 100.0 ppm lead (as $PbCl_2$) or cadmium (as $CdCl_2$).

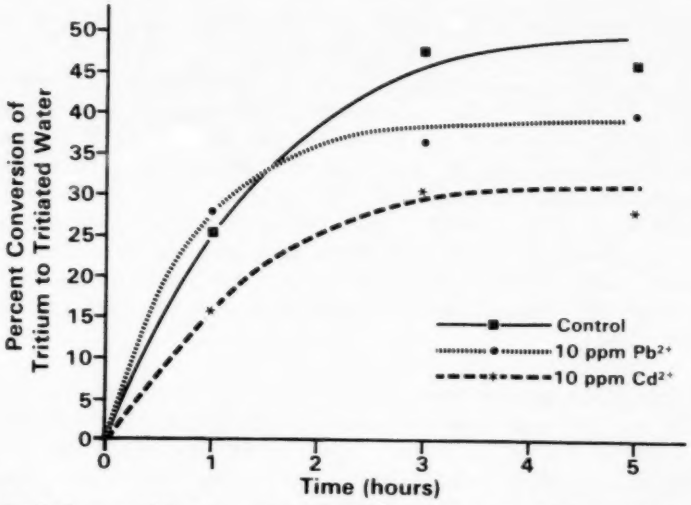

Figure 10. Tritium oxidation by *Alcaligenes paradoxus* after 40 minutes pretreatment in solution of 30 mM Tris, pH 8.0, and 10^{-2}M EDTA followed by washing and resuspension in distilled water amended with 0.0 or 10.0 ppm lead (as $PbCl_2$) or cadmium as ($CdCl_2$).

Other investigators found that EDTA produces structural changes in the outer membrane of *Escherichia coli* (Bayer and Leive, 1977) and a variety of other gram-negative bacteria (Gray and Wilkinson, 1965b). EDTA has also been found, especially when used in conjunction with Tris buffer, to stimulate the rate of lysis of gram-negative organisms by lysozyme (Repaske, 1956; Repaske, 1958; Goldschmidt and Wyss, 1967; Voss, 1964). Gray and Wilkinson (1965a), also showed that EDTA alone has direct bacteriocidal action against *Pseudomonas aeruginosa* and *Alcaligenes faecalis*. Thus, it was decided to use a combination of some of these techniques on *A. paradoxus* with the hope of increasing its sensitivity to lead and cadmium.

The results of the EDTA-Tris treatment of *A. paradoxus* cells can be seen in Figures 10 and 11. Both the 40-minute and the 2-day treatment produced a definite reduction in the extent of tritium oxidation while using only 10.0 ppm cadmium and lead. In both cases also, the effect of lead was less than that of cadmium by an apparently similar percentage. This was a considerable difference from the earlier results and showed that it is definitely possible to modify the activity of *A. paradoxus* to make it more suitable, at least in this case, for detecting the desired low levels of certain compounds.

This shows that preliminary studies indicate it is feasible to chemically alter the response of *A. paradoxus* to show desired effects, in this instance by increasing its sensitivity to two heavy metals. Considerable work still needs to be done to clearly define the types of conditions that can cause the effects observed and to standardize procedures, but the potential for sensitizing *A. paradoxus* to these and perhaps lower levels of lead and cadmium exists.

In the course of this work it was found that there were soils available that had been amended with cadmium in 1973. As shown in Table 5, the levels of cadmium in the soils at the time of these studies were near background levels, and in view of the just-described results with cadmium, no response from *A. paradoxus* to such low levels was expected. However, as can be seen in Figure 12, there was a distinct difference in the oxidizing ability of *A. paradoxus* in soil 1 compared to soil 4. Soils 2 and 3, although not shown, were also below soil 1 in the extent and rate of their reaction. They are not shown because the data did not allow lines to be drawn through their points with sufficient confidence to justify adding them to the graph. This was not the case with the data presented. The fit of the lines to the points was good.

The question now arises as to what was causing the effect. The cadmium levels are so low and similar that it is doubtful that the cause was due to cadmium. More likely it seems that there was some residual effect on the soils from the cadmium additions. The only difference in the soils was supposedly the amounts of cadmium added in 1973, so the possibility of other differences inherent in the soils can be excluded. Plant studies run in 1975 also showed poorer growth on soil 4 compared to soil 1 (A. G. Wollum, personal communication), although admittedly the cadmium levels were higher then.

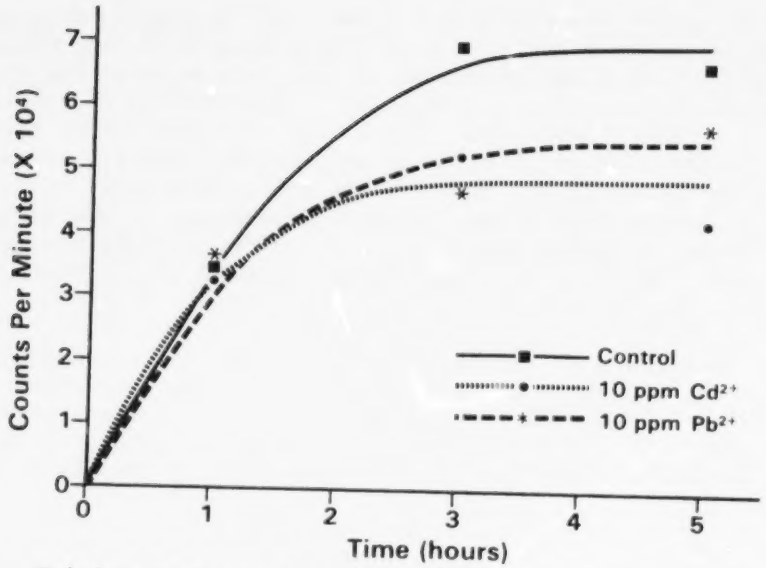

Figure 11. Tritium oxidation by *Alcaligenes paradoxus* after 48 hours pretreatment in a solution of 30 mM Tris, pH 8.0, and 10^{-2}M EDTA followed by washing and resuspension in distilled water amended with either 0.0 or 10.0 ppm lead (as $PbCl_2$) or cadmium (as $CdCl_2$).

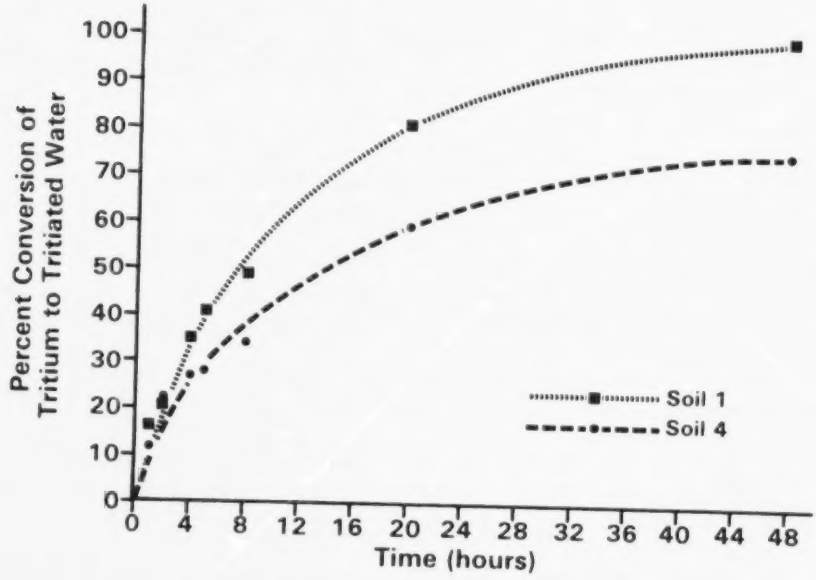

Figure 12. Tritium oxidation by *Alcaligenes paradoxus* after inoculation into otherwise sterile soils that were amended with no cadmium (soil 1), or 20 ppm cadmium (as $CdCl_2$) (soil 4) in 197 and now have levels of 0.18 and 0.28 ppm cadmium respectively.

Whatever caused the effect may or may not be found. However, the differences in the rate and extent of the tritium oxidation between the two soils is very interesting and would certainly seem worthy of further investigation, especially in light of the somewhat similar research findings of McFarlane et al. (1978). They found that identical soils fumigated with varying concentrations of sulfur dioxide (SO_2) for 1 and 2 years showed differences in their ability to carry out the oxidation of tritium by their indigenous microbial populations. These differences in the ability to carry out the oxidation reaction were found to be a function of the degree of SO_2 fumigation even though no residual SO_2 was present in the soils at the time of the tests.

These findings, even though done with unidentified hydrogen-oxidizing microorganisms, indicate once again that even though virtually no detectable chemical differences can be found between soils, biological effects, viz., the ability to oxidize tritium by microorganisms, can be observed. What these results mean and why they occur are intriguing questions awaiting future research.

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